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(54) Title: TRANSFECTION USING PLASMID PREPARATIONS

(57) Abstract: There is provided a method for plasmid DNA extraction to reproducibly yield relatively crude preparation which is suitable for transfection into mammalian cells. The inventors have determined that the high level of purification previously thought to be necessary in order to successfully allow transfection of the plasmid DNA into cells is not required. The inventors have developed a method of producing a transfectable plasmid preparation that can be performed on a large scale that avoids the use of specialized equipment and reagents.

Transfection Using Plasmid Preparations

The present invention relates to the transfection of cells using plasmid preparations, and to related materials, uses and methods, including plasmid purification methods.

The large-scale manufacture of plasmid DNA is important for the field of gene therapy (see e.g. US 5,561,064) and for production processes which require large quantities of DNA, e.g. for transient transfection of mammalian cells for recombinant protein production and virus vector production. It is considered that for effective transfection of eukaryotic cells, plasmid preparations must be highly purified (see eg Jordan et al. 1996; Heilig et al., 1998). Moreover, to be used for in vivo gene therapy applications, the final DNA for formulation needs to be of an extremely high quality in order to be approved for human use by regulatory agencies.

The criterion "suitable for transfection" is judged by the majority of researchers by the visual appearance of a DNA preparation on an agarose gel after electrophoresis. As well as providing approximate molecular weight determinations, electrophoresis can also reveal the presence of a significant bacterial genomic DNA contamination, degradation of plasmid DNA or the presence of RNA in the preparation. Furthermore, the conformational properties of plasmid DNA can be evaluated: a differentiation between open circular, linear or supercoiled forms can be seen in most preparations. The majority of plasmid DNA is usually

found in the native supercoiled form, which migrates aster than the other, more extended forms of plasmid DNA.

Much literature describes methods for achieving adequate levels of plasmid purity. However, most plasmid purification methods involve an initial alkaline lysis step, followed by precipitation of cellular debris and then purification of the plasmid DNA from the resultant supernatant. For example, at laboratory scale (up to about 10mg of DNA per preparation) pure preparations are produced from crude material mainly by two processes.

The principal method of purification to separate plasmid DNA from host cell contaminants is the use of disposable anion exchange columns, such as those produced by Qiagen and Macherey & Nagel. Clarified cell lysate is loaded onto the column and DNA binds to the matrix via the charged phosphate groups on the phosphodiester backbone. After subsequent washes to remove proteins and RNA, the application of an increased salt concentration and/or alteration of the pH in the mobile phase achieves separation of DNA from the stationary phase. This is followed by precipitation of the eluted DNA, typically with isopropanol, and a wash step in 70% ethanol.

A more traditional method, and until the introduction of these columns the most commonly applied method for DNA purification, relies on the separation of DNA in a salt (caesium chloride) density gradient by ultracentrifugation. DNA forms a band at a point in the centre of the tube (and the gradient) corresponding to its density. It can be identified by fluorescence if the gradient is carried out in the presence of ethidium

bromide. Two gradients in series are typically used in order to produce a highly pure DNA preparation. After removal of the plasmid fraction, ethidium bromide is extracted with an organic solvent (typically butanol). Precipitation with alcohol (such as isopropanol or ethanol) followed by a wash step in 70% ethanol is used to remove the butanol and concentrate the DNA to the required concentration.

Other additional stages may be added in order to increase purity and ease the purification method according to different interpretations of these protocols.

These two purification methods have certain drawbacks, which make their use prohibitive to produce DNA for transfection at scale. Single use chromatographic columns are impractical and costly for large-scale plasmid preparation. Additionally, column chromatography is a complex discipline, requiring great skill to achieve good purification. Density gradient ultracentrifugation methods are also impractical for large-scale DNA production, as they use relatively high concentrations of ethidium bromide, a powerful carcinogen, as well as caesium chloride and butanol. Moreover long run times are required at very high centrifuge speeds (50,000 - 90,000 rpm) in order to correctly form the salt density gradient. In addition, there is a limitation in the volume which can be used per rotor in a centrifuge run and the use of larger volumes also means that longer run times are needed at slower speeds in order to form an adequate gradient for the separation of plasmid DNA.

Other common laboratory purification techniques are also unsuitable for large scale purification, e.g. phenol-chloroform extraction, which involves the use of a carcinogen.

Most of the large-scale processes described in the literature and in previous patents are designed to produce highly purified plasmid DNA preparations, with the specific goal of reaching a product suitable for in vivo gene therapy and the stringent requirements of regulatory bodies. This includes meeting requirements related to genomic DNA, host cell protein, RNA and LPS (lipopolysaccharaides). They are thus complex multi-stage operations. The following references provide an overview of some of the currently applied industrial processes, (Marquet et al (1995), Horn et al (1995), Prazeres et al (1999). The majority of such large-scale processes also consist of a primary extraction stage (again alkaline lysis is the preferred method) and various combinations of chromatographic, filtration, and precipitation steps.

Wurm F. M. and Bernard A. (1999) review large-scale transient transfection technology. Transient approaches offer a means to produce recombinant proteins rapidly at scale in animal cells and avoid the lengthy processes associated with the development of stable cell lines. The production of recombinant (r-) proteins in a short time is gaining increasing importance, particularly with the increase in the number of proteins that need to be produced at the gram scale for further study or evaluation. However, efficient transfection of cells in a 50l stirred tank requires 50-100 mg of plasmid DNA. The need for large quantities of highly purified DNA is

identified in this review as the most significant drawback to achieving transfection at scale.

There remains a need therefore for plasmid preparations suitable for transfection into cells (especially eukaryotic cells) on a large scale and for plasmid purification methods to yield such preparations.

Broadly, the present inventors have developed a method for plasmid DNA extraction to reproducibly yield a relatively crude preparation, which they have surprisingly found to be suitable for transfection into mammalian cells, despite prevailing opinion in the art that high purity is required for transfection into eukaryotic cells.

To our knowledge only two reports of the transfection of a crude plasmid preparation exists in the open literature, namely Tatsuka et al. (1995) and Song et al (1999). In the Tatsuka et al paper, plasmids were "partially purified" on a small miniprep scale by alkaline lysis, phenol-chloroform extraction and ethanol precipitation only. These preparations were transfected in BALB/c 3T3 cells by electroporation; parallel transfections with "pure DNA" (prepared by 2 x CsCl density gradient ultracentrifugation) served as a comparison. Crude plasmid preps were found to be toxic to the cells, whereas cells electroporated in the presence of pure DNA were viable. A medium exchange, 1 hour post-transfection, overcame the cytotoxicity problem. It is to be noted that the use of miniprep purification methods and the use of phenol-chloroform extraction make the purification method of Tatsuka et al.

wholly unsuitable for scaling up, on both economic grounds (i.e. because of the use of disposable miniprep hardware) and safety grounds (phenol is an irritant and a potent carcinogen). Miniprep purification is impractical for plasmid purification on a scale greater than a few tens of micrograms.

The Song et al paper describes the preparation of DNA at small scale by a direct lysis and precipitation method to rapidly obtain crude DNA preparations. The principle contaminant in their preparations was described as genomic DNA. However, analysis of the agarose gel provided in the paper also shows that visible amounts of RNA were co-purified. The authors used the commercially available Effectene transfection reagent and a plasmid encoding GFP to transfect HeLa cells, which displayed 20-30% transfection efficiency.

In contrast, more literature suggests that nothing but highly purified plasmid DNA is suitable for the transfection of animal cells. See e.g. Weber et al (1995), Fuhrman et al (1998). Similarly the transfection guide of Promega states that "The quality of the DNA used for transfection is critical. Purified plasmid DNA should be free from protein, RNA and chemical contamination" and Qiagen's guide to transfection states "Transfection experiments yield best results when plasmid DNA of the highest purity is used."

The purification method described in the examples involves alkaline lysis followed by a series of easily performed precipitations, all of which can be conducted on a large scale. Plasmid DNA stays intact even when

subjected to relatively harsh mixing conditions in a stirred reactor vessel during alkaline lysis, a process previously thought to damage plasmid DNA unless conducted without vigorous mixing. The method offers significant benefits in that it may be performed at large scale and requires little specialized equipment or reagents. In addition it is quick and cost effective. It significantly benefits large-scale commercial plasmid production, e.g. as a step in protein expression by transient transfection. At present, preparations of 50-100 mg DNA can typically be prepared in about 3 hours by the method of the invention. It is expected that the method can be scaled up further without difficulty in larger reaction vessels. Moreover, it is expected that the particular method described could be varied to some extent, whilst still producing transfectable, yet relatively crude, plasmid preparations.

Accordingly, in a first aspect, the present invention provides a plasmid DNA preparation which is transfectable into eukaryotic cells, the plasmid preparation containing at least about 10 mg DNA, wherein the preparation is a crude preparation containing RNA and/or other cellular components, e.g. protein, LPS and chromosomal DNA.

RNA may be present in an amount visualisable by agarose gel electrophoresis, e.g. by ethidium bromide staining. RNA may be present in the preparation in a ratio to DNA (preferably plasmid DNA) within a range having a top end selected from 20:1, 10:1, 5:1, 4:1, 3:1 and 2:1 and a bottom end (independently) selected from 1:20, 1:10, 1:5, 1:4, 1:3 and 1:2. In particular, RNA may be present in an amount approximately equal to that of the DNA.

Protein if present is preferably in an amount not visualisable by gel electrophoresis, e.g. by Coomassie and/or silver staining. Protein may be present in an amount greater than about 0.05 g per litre of plasmid preparation, or greater than about 0.1 g/l, 0.15 g/l, 0.2 g/l, 0.25 g/l, 0.3 g/l or 0.35 g/l and may be in an amount lower than about 1 g/l, 0.75 g/l, 0.5 g/l or 0.4 g/l.

Preferably the preparation contains at least about 15 mg, 20 mg, 25 mg, 30 mg, 40 mg, 50 mg, 75 mg, or 100 mg DNA (more preferably plasmid DNA). Preferably the amount of plasmid DNA in the preparation is equivalent to the plasmid in at least 2l, 3l, 5l or 10l of fermentation broth. However, it is thought that the invention may be scaled up even further, e.g. to an industrial scale involving grams of DNA and hundreds of litres of fermentation broth.

In a second aspect, the invention provides the use of a plasmid preparation according to the first aspect for the transfection of a eukaryotic cell and a method of transfecting a eukaryotic cell, the method comprising exposing the cell to a plasmid preparation according to the first aspect under conditions suitable for transfection to occur.

Transfection may be by any suitable technique, e.g. calcium phosphate co-precipitation, electroporation (see Example 7 below) or lipofection. However, for practicality and economy in large-scale transfection, calcium phosphate co-precipitation is preferred. Electroporation in particular generally achieves only low

transfection levels and cannot be conveniently performed on a large scale without expensive specialised equipment. However, it has been shown that eukaryotic cells can successfully be transfected by electroporation using the crude preparations of the invention.

It has been found that the precipitate in the calcium phosphate method can be toxic to eukaryotic cells (this applies also to highly pure plasmid preparations). To address this, it may be desirable to change or dilute the medium of the transfected cells following transfection. Medium exchange or dilution a short time after transfection promotes cell survival, whereas delaying medium exchange/dilution promotes efficient transfection. Suitable times for medium exchange/dilution may be from about 1-5 hours post-transfection.

Medium exchange/dilution may alleviate toxic effects of contaminants in crude plasmid preparations, particularly when electroporation is used.

Preferred cells are higher eukaryotic cells, more preferably animal cells, still more preferably mammalian cells, most preferably HEK293 cells.

In a third aspect, the invention provides a method of preparing from bacterial cells a plasmid DNA preparation containing at least about 10 mg of DNA, the preparation being suitable for transfection into eukaryotic cells, the method comprising the steps of:

(a) lysing the bacterial cells;

(b) precipitating bacterial cell debris;

(c) separating the cell lysate, containing plasmid DNA, from the precipitated cell debris;

(d) precipitating the plasmid DNA from the cell lysate, separating the precipitated plasmid DNA from the cell lysate and resuspending the plasmid DNA; and optionally

(e) selectively precipitating RNA and/or cell protein and/or LPS from the cell lysate and/or resuspended plasmid DNA and separating the supernatant, containing the plasmid DNA, from the precipitate.

Preferably at least two steps (d) are performed, more preferably one before and one after step (e).

Using the method of the invention, large amounts of plasmid DNA preparation (e.g. containing at least about 10 mg, 20 mg, 30 mg, 50 mg, 75 mg, or 100mg DNA, particularly plasmid DNA) may be produced without the need to combine DNA from different batches of cell lysate or from eluted fractions of different column chromatography steps. Thus parallel processing of different plasmid DNA batches intended for the same plasmid preparation can be reduced, simplifying purification.

Preferably step (a) is an alkaline lysis step, which may conveniently be carried out in a stirred vessel. However, other methods of cell lysis may be used, e.g. as disclosed in Heilig et al. 1998. Lysozyme will generally be absent in the alkaline lysis step.

Preferably step (b) is precipitation using high molar salt solution, preferably under acidic conditions. Most preferably the precipitation solution comprises about 3M potassium acetate (KAc) and acetic acid. Repetition of this step (in particular using 3M KAc / acetic acid) has been found not significantly to affect transfection efficiency of the resultant plasmid preparation, so is preferably not performed.

Preferably step (c) is a filtration and/or centrifugation step.

The precipitation of step(s) (d) may use isopropanol, although alternatively ethanol or PEG can be used (see e.g. Heilig et al. 1998 for protocols using PEG). PEG has the advantage of non-flammability, reducing the hazard presented by alcohol in large-scale purification. The use of alcohol on an industrial scale usually necessitates explosion-proof equipment, which can be expensive. PEG avoids this problem.

The selective precipitation of steps (e) preferably uses high molar salt solution, preferably ammonium acetate, preferably at about 3M.

Preferably bacterial cells during the lysis step are in a volume of at least about 250ml, more preferably at least about 500 ml, more preferably at least about 1 l, 2l or 3l. Such cells may have been derived from a greater or lesser volume of cell culture solution. It has surprisingly been found that alkaline lysis can be conducted on such large volumes to yield transfectable plasmid.

Preferably the yield of the method is at least about 5 mg DNA (particularly plasmid DNA) per litre of cell culture solution, and may be at least about 10, 15 or 20 mg/l. The yield is preferably at least about 0.25 mg DNA (particularly plasmid DNA) per gram of bacterial cells (wet weight), and may be at least about 0.5, 0.75 or 1 mg/g.

The method may comprise the additional step of treating the plasmid preparation to further remove RNA, particularly when RNA levels are high relative to plasmid DNA levels. Preferably the treatment is with RNase, suitably RNase A. It has been found that high levels of RNA can reduce transfection efficiency in eukaryotic cells.

The inventors have found that crude "RNA containing" DNA extracts are highly transfectable in HEK293 cell line using, for example, the calcium phosphate method. Under optimal condidtions, in both adherent and suspension-adapted modes of culture, such extracts achieve highly efficient transfection and result typically in equivalent levels of transient recombinant protein expression as achieved upon transfection of pure DNA preparations.

Considering the data from the majority of transfections performed at the optimal DNA concentration (2.5µg/ml culture volume) and a 1 minute precipitation time, bacterial RNA in most DNA extracts appears to have little effect on the transfectability of plasmid preparations using, for example, the calcium phosphate method and the HEK293 EBNA cell line. Only when RNA contaminants exceed

a certain value is a decrease in expression observed. The transfectability of such extracts in this system can however be restored by RNase digestion. Transfection of a DNA preparation containing RNase has been determined by the inventors to yield the same result as the transfection of a non-RNase containing preparation. Therefore, it has been surprisingly determined that the enzyme need not be removed from the DNA preparation before transfection.

"High" RNA levels in this context may be defined as RNA being present in the preparation at least in excess (by weight) compared to the DNA in the preparation, particularly compared to the plasmid DNA, or it may be defined as RNA being present in at least 2-, 4-, 8-, 12- or 16-fold excess. The tolerance of cells for RNA will depend on cell type, transfection conditions (e.g. adherent or suspended cells) and transfection method (e.g. electroporation, calcium phosphate co-precipitation or lipofection).

Thus, it has been surprisingly determined that transfection of a eukaryotic cell can take place in the presence of all contaminants contained in the plasmid preparation. It has been shown that it is unnecessary to remove any or all of the contaminants in order to successfully transfect a cell with the plasmid of the plasmid preparation. In other words, the presence of contaminants does not substantially affect the transfectability of the plasmid.

If it is felt desirable to decrease the RNA content in the crude plasmid preparation, this can be done as

mentioned above using RNase. However, as demonstrated herein, it is surprisingly not necessary to remove the RNase contaminant from the plasmid preparation prior to the transfection step. Thus, transfection takes place in the presence of the plasmid preparation contaminants e.g. RNA and/or RNase.

Preferably the method is carried out in the absence of any or all (preferably all) of: chromatography, concentration in a density gradient and phenol-chloroform extraction. It was formerly thought necessary to carry out one or more of these steps to obtain a plasmid preparation of sufficient purity to be suitable for transfecting eukaryotic cells.

However, the method may also include other purification steps which are suitable for large scale purification (and preferably industrial scale purification), such as dialysis (which may for example remove salts and/or small fragments of nucleic acid) and/or filtration, e.g. ultrafiltration.

The present invention also provides plasmid preparations prepared according to, or as preparable by, the above method. Such preparations may be as defined in the first aspect.

It has been found that such plasmid preparations are crude, i.e. a considerable quantity of RNA remains in the DNA preparation after the extraction procedure (though this will be reduced if RNase A is used in the method). The preparation may also contain LPS, host cell protein, and genomic DNA. However the plasmid itself appears to

be intact and mainly supercoiled and bears a close resemblance to that of "pure" plasmid DNA prepared by a standard laboratory technique (see figure 3).

In a fourth aspect, the present invention provides a method for transfecting eukaryotic cells, having previously produced a plasmid preparation according to the method of the third aspect, the method comprising transfecting the cells with said plasmid preparation such that transfection takes place in the presence of contaminants contained in the plasmid preparation, e.g. RNA and/or RNase. Similarly the invention provides the use of said preparation for transfecting eukaryotic cells.

In a fifth aspect, the present invention provides the use of RNase to remove RNA from, or to reduce RNA levels in, a crude plasmid DNA preparation (e.g. as defined in relation to the first aspect) prior to transfection into a eukaryotic cell. Such use may be without subsequent removal of the RNase prior to transfection as discussed above. Preferred RNase is RNase A. Such use may be without treatment to inactivate or remove RNase from the preparation prior to transfection. This has surprisingly been found unnecessary, despite the possibility of RNase entering the transfected cells and disrupting RNA metabolism therein. This surprising finding is particularly advantageous, as RNase is highly stable and difficult to inactivate.

In further aspects the present invention provides cells transfected according to the above methods and/or transfected with the plasmid preparations above and/or

with plasmid preparations produced according to the above methods. Furthermore, the invention provides a process for producing a protein, the process comprising culturing such a cell under conditions under which protein expression can occur. The process may include a step of isolating, concentrating and/or purifying the protein thus expressed. The protein may be formulated as a commercial product, e.g. an enzyme or pharmaceutical. Such further aspects are within the capability of the skilled person, assisted if necessary by standard reference texts such as Sambrook et al (1989) Molecular Cloning 2nd ed, Cold Spring Harbor Laboratory Press and Ausubel et al (1992) Short Protocols in Molecular Biology 2nd ed, John Wiley & Sons (or later editions of these texts).

For the avoidance of doubt, it is hereby explicitly stated that all preferred features apply to all aspects of the invention, except where clearly incompatible.

Alkaline lysis

Alkaline lysis, described first by Birnboim & Doly (1979) is the most widely used method to extract and separate plasmid DNA from bacteria, e.g. E. coli. In typical alkaline lysis methods, which are preferred in the purification method of the invention, the breakdown of the bacterial cell envelope is elicited first by the action of EDTA in a resuspension buffer, which acts as a chelating agent binding to divalent cations which stabilise the LPS (lipopolysaccharaide) structures on the outer membrane. SDS, the principal lysis agent, acts to solubilise the inner cell membrane and probably also on the peptidoglycan layer between the inner and outer

membranes. NaOH causes the pH to increase rapidly to between 12.0 and 12.5, this causes the denaturation of DNA (defined as strand separation, i.e. the breaking of hydrogen bonds between bases). In fact, in a pH of 12.78 has been used in one of the extractions during the lysis step. However, it is unadvisable to increase the pH to above pH 13 as this may irreversibly denature the plasmid molecule and thereby reduce significantly the efficiency of the subsequent precipitation step in which plasmid DNA correctly renatures (with a pH shift to approximately pH 5.0). Addition of acidic KAc results in the formation of a large precipitate which includes cell debris, chromosomal DNA and complexes formed between SDS and proteins. The relatively large bacterial chromosomal DNA mass (in E.coli, 4 million base pairs) irreversably denatures and co-precipitates, with this drop in pH. Plasmid DNA (3 - 15 thousand bp) correctly renatures and remains soluble in the liquid phase. After these primary extraction stages, the liquor is separated from the precipitate, usually by centrifugation, although filtration is a further possibility.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated by reference.

Figure 1. Stirred tank reactor for the process of alkaline lysis. Resuspended cells and Lysis solution are mixed online and then in the reactor at stirrer speed 500

rpm. Addition of the preparation solution is via the sparger at a stirrer speed of 500 rpm.

Figure 2. Alkaline lysis at the 1.5l scale in a stirred tank reactor. (a). Lysis, (b). Addition of precipitation solution, (c). Precipitation, (d). Clarified lysate (after centrifugation and passage over a folded filter).

Figure 3. Comparison of DNA preparations produced by method described in invention and Macherey & Nagel column method, 1 % agarose gel. All plasmids pEGFPN1 (4.7 Kb), 1µg DNA/well.

Figure 4. Transient GFP expression from attached HEK293 EBNA cells after transfection of different preparations of pEGFPN1 by the calcium phosphate co-precipitation method. "Pure" refers to DNA preparations purified by a kit method (Macherey & Nagel, AX 10,000), whereas "crude" refers to partially purified DNA prepared and described. Transfection conditions as described in example 3. All bars represent the mean of 3 transfections, in 12 well plate format, 2.5 µg DNA per well, cells seeded 18 hours before transfection at 1 x 10^5 cells/ml.

Figure 5. Transient GFP expression 3 days post transfection from HEK293 EBNA cells in suspension after transfection of different preparations of pEGFPN1 by the calcium phosphate co - precipitation method. "Pure" refers to DNA preparations purified by a kit method (Macherey & Nagel, AN 10,000), whereas "crude" refers to partially purified DNA prepared as described. Transfection conditions as described in example 4. All bars represent the mean of 3 tranfections, in 12 well

plate format, 2.5 µg DNA per well, cells seeded 2 hours before transfection at 5 x 10^5 cells/ml.

Figure 6. Transient GFP expression 4 days post transfection from HEK293 EBNA cells in stirred tank reactor upon transfection of different preparation of pEGFPN1 by the calcium phosphate co-precipitation method. "Pure" refers to DNA preparations purified by a kit method (Macherey & Nagel, AN 10,000), whereas "crude" refers to partially purified DNA prepared as described. Transfection conditions as described in example 5. All bars represent the mean of 3 measured wells, in 12 well plate format, 1.5 mg DNA per reactor, cells seeded 2 hours before transfection at 5 x 10^5 cells/ml.

Figure 7. The effect of increasing amounts of bacterial RNA on the transfectability of pure DNA in HEK293 EBNA cells using the Calcium Phosphate co precipitation method. Transfection in 12 well plate. 3 transfections repeated per preparation, 2.5 µg pEAKeGFP applied per well. RNA preparation extracted from the clarified lysate by isopropanol precipitation, ammonium acetate precipitation, resuspension of the ammonium acetate precipitate, dilution and a final precipitation with isopropanol. RNA quantitated using the ribogreen reagent (Molecular probes). Details of transfection are outlined in examples 3, 4 and 6 of the invention.

Figure 8. The effect of increasing amounts of bacterial RNA on the transfectability of pure DNA encoding anti Rhesis D IgG in HEK293 EBNA cells using the Calcium Phosphate co precipitation method. Transfection in 12 well plates. 3 transfections repeated per preparation,

2.5µg DNA (LH1 (light chain) : LH2 (heavy chain), 30:70) per well. RNA preparation extracted from the clarified lysate by isopropanol precipitation , ammonium acetate precipitation, resuspension of the ammonium acetate precipitate, dilution and a final precipitation with isopropanol. 8a. Adherent (12 well plate), 8b. Suspension (12 well plate). Details of transfection are outlined in examples 3, 4 and 6 of the invention. Anti RhD IgG measured 5 days post transfection by ELISA.

Figure 9. The effect of differences in the DNA preparation used during transfection upon the transient expression of anti RhD IgG in HEKEBNA adherent cells. Expression of anti RhD IgG. Days post transfection. Co-transfection of two plasmids, LH1 and LH2, encoding the light and heavy chains of anti RhD IgG. LH1 and 2 were transfected in a 30:70 ratio at 2.5 µg DNA per well using various compositions of pure and crude preparations of plasmid. Crude LH1 contained 1.2µg RNA/µg DNA and crude LH2 3.52 µg DNA. Corresponding values of RNA are represented on the graphic per transfection. Transfection as described in example 3, IgG measured in supernatant by ELISA. crRNase, crude preparations treated with RNase before transfection.

Figure 10. The effect of differences in the DNA preparation used during transfection upon the transient expression of anti RhD IgG in HEKEBNA suspension cells. DNA transfected at 2.5µg/well. 12 well plate format (as example 4.) LH1: LH2 transfected 30:70. Pure (Macherey-Nagel kit purified), crude (partially purified by method described in examples 1 & 2.) crRNase (crude treated with RNase A). Transfections as described in example 4.

Figure 11. Restoration of transfectability to a preparation of pEAKeGFP by treatment with RNase in adherent HEKEBNA cells (transfection conditions as example 3.) All transfections represent the mean fluorescence values from three wells transfected at the same time. DNA amount/well (a). 1.0 µg/well, (b). 2.5 µg/well.

Figure 12. Comparison of DNA preparation after extraction. 1 % agarose gel electrophoresis. M-1kb ladder. 1 - Pure pEGFPN1 (Macherey & Nagel), 2 - Crude prep. 1. pEGFPN1, 3 - Crude prep. 2. pEGFPN1, 4 - Crude prep. 3. pEGFPN1. All lanes 1µg with respect to plasmid DNA.

Figure 13. Evaluation of precipitate formation by turbidimetric (OD320nm) onset (13a) and evaluation of nucleic acid concentration in solution by spectrophotometric measurements (A260nm) before and after calcium phosphate precipitation (13b).

Figure 14. Comparison of transient GFP expression 3 days post transfection in adherent (14a) and suspension adapted (14b) HEK293EBNA cells. 2.5 µg/ml pEGFPN1 transfected per ml culture volume. GFP fluorescence is measured in the 12 well plate in-situ. Values presented as relative fluorescence units (rfu). Each bar represents the mean of 3 transfections. Suspension adapted cells were lysed prior to measurement.

Example 1: Large-scale alkaline lysis

(a) Cell harvesting

E. coli cells (DH5a harbouring the plasmid pEGFPN1) were harvested from a 12l fermentation at an OD600 of 20 or more, typically achieved within 12 hour of inoculation. The culture was grown at 37°C and under air saturation in a 20l bioreactor (ISF200, INFORS, Bottmingen, Switzerland), stirrer speed 600rpm, air flow rate 10l / min, in batch mode on a semi-defined glycerol based medium. Cells were harvested by recovering the fermentation broth from the reactor and centrifuging in 750ml flasks at 4,000g for 10 minutes. Cells from 2.5l of fermentation broth were resuspended in 200ml of 50mM glucose, 25mM Tris, 10 mM EDTA, pH 8.0 and frozen immediately at -20°C.

(b) Large-scale alkaline lysis (shown schematically in Fig. 1)

Frozen cells in suspension from 3l fermentation broth, were thawed and resuspended further to a final volume of 500ml in chilled resuspension buffer (see step (a)). Alkaline lysis was carried out in a 2l stirred tank reactor fitted with two rushton turbines (Applikon, Dietikon, Switzerland), in which pH and temperature were monitored, figure 1. Cells and lysis solution (1% SDS, 0.2M NaOH) (500mls) were mixed on-line in a 1:1 ratio (500ml:500ml) by pumping the two solutions at the same flowrate over two peristaltic pumps into a single tube (total flowrate of combined stream, 140ml/min). This tube reaches the base of the reactor, where mixing is

continued by the action of the agitator at a stirrer speed of 500rpm. After complete addition of the combined lysate stream to the reactor (7 minutes), the lysis process was continued in the reactor for a further 5 minutes (stirrer speed 500rpm). The pH during lysis was between 12.0 and 12.8. Precipitation with 500ml chilled 3M potassium acetate / acetic acid solution was carried out in the reactor under the same conditions. The solution was added via the sparger under the same conditions (stirrer speed, 500 rpm; addition time, 7 minutes), pH after precipitation was verified to be 5.0. After a further period of mixing (500 rpm, 10 min) the lysate was removed from the reactor. Figure 2 shows the physical appearance of the different stages in this process.

The contents of the reactor were emptied into 2 x 750ml centrifuge pots (flat base) which were then centrifuged at 4,000g, 4°C for 30 minutes (Heraeus Varifuge 3.0R, Kendro laboratory products, Geneva, Switzerland). The supernatant was clarified further by a single passage over a coarse folded filter (Macherey - Nagel, Oensingen, Switzerland). Liquid recovery was typically 1.2 - 1.3l from 1.5l of starting materials.

Example 2. Partial purification of plasmid DNA from clarified lysate by precipitation only.

Plasmid DNA was extracted from the clarified liquor by the addition of 0.6 volumes isopropanol, after mixing and incubating at room temperature for 10 minutes the precipitate was extracted by centrifugation at 4,000g for 20 minutes. After removing the supernatant, crude DNA

pellet was resuspended in a total volume of 200ml TE, pH 8.0, (50mM Glucose, 25mM Tris, 10mM EDTA). Solid ammonium acetate was added to 2 x 100ml of the resuspended DNA pellet to reach a concentration of 2.5M. This was mixed rapidly and incubated on ice for a period of 5 minutes, after which it was centrifuged at 4°C, 4,000g for 5 minutes. The DNA containing supernatant was precipitated directly with 0.6 volumes of isopropanol at room temperature (incubation and centrifugation conditions as previously). The supernatant was discarded and the resulting pellet washed in 70% ethanol, after centrifugation excess liquid was removed. All precipitation and centrifugations were performed in 250ml polypropylene conical base centrifuge tubes (Corning, NY). The pellet was left on the bench to partially dry and subsequently resuspended in a small volume (20ml) of TE and analyzed. DNA was quantified using the picogreen reagent (Molecular Probes, Leiden, Netherlands) in microtiter plate format using a lambda DNA standard (Molecular Probes). Before quantitation preparations were digested with RNase, followed by digestion with a single site restriction enzyme (Hind III/EcoRI). This preparation was diluted to give a DNA concentration of 1 µg/µl. Similarly RNA concentrations were determined after DNase digestion, followed by quantification with the ribogreen reagent (Molecular Probes).

Two preparations (1 and 2) prepared by this extraction method (each extracted from 3l of fermentation broth) are shown after electrophoresis on a 1% Agarose gel in the presence of ethidium bromide (figure 3). Typical yields from 3 similar preparations of pEGFPN1 were 93mg plasmid DNA (prep 1), 65mg plasmid DNA (prep 2) and 73mg plasmid

DNA (prep 3, not shown on the gel). Preparations 1 and 2 contain approximately the same concentration of RNA as DNA (prep 1 contains RNA at 1.15 µg/ml, prep 2 1.19 µg/ml). Protein bands were not detectable either by Coomassie staining or the more sensitive silver staining, (preparations were treated with RNase and DNase, as both are detected with silver stain).

Using the method described we routinely obtain 20 - 30mg of extractable DNA per liter of fermentation broth, this corresponds to approximately 1mg extractable DNA / g wet weight cells.

Example 3. Transfection by the calcium phosphate co - precipitation method of partially purified plasmid DNA in a HEK293EBNA adherent cell culture to produce recombinant protein, using Green Fluorescent Protein (GFP) as a reporter.

Partially purified preparations of plasmid, pEGFPN1 (Clontech), prepared by the method described in examples 1 & 2, were transfected in comparison to "pure" DNA preparations. "Pure" DNA was prepared by a commercially available column kit method (AX 10,000, Macherey & Nagel) after cultivating E.coli in shake flasks using LB medium to a density equivalent to OD600 3 - 5. Figure 3 shows the appearance of partially purified and pure preparations on a 1% Agarose gel.

HEK293 EBNA cells were cultivated in T flasks in DMEM + 2% fcs and passaged upon reaching confluence. 20 hours prior to transfection cells were seeded in 12 well plates at 1 x 10^5 cells/ml/well. For each series of

transfections, 10 µg pEGFPN1 (Clontech) was added to 200 µl 2 x $CaCl_2$ (250mM) this was mixed by pipette with an equal volume (200 µl) of 2 x Hepes phosphate buffer (140mM NaCl, 50mM Hepes, 1.4mM NaH_2PO_4, pH 7.05.). After a 1-minute incubation time, 100 µl of precipitate was added to each of 3 wells containing cells in 1 ml of medium. Medium was exchanged 4 hours post transfection in order to avoid problems associated with the toxicity of the precipitate. GFP (an intracellular protein) fluorescence was measured non - invasively directly in the 12 well plate using a fluorescence plate reader (excitation filter 488nm, emission filter 520nm).

A comparison of the transfectability of 2 partially purified preparations (preps 1 & 2) manufactured by the method described was carried out in comparison to preparations purified by the method of Macherey & Nagel. The expression over 3 days from parallel transfections using different preparations is illustrated in figure 4. There is no difference in GFP expression, between cells transfected with partially purified plasmid and those transfected with a preparation of "pure" DNA, despite the presence of an equivalent concentration of RNA to DNA in the transfection.

Example 4. Transfection by the calcium phosphate co - precipitation method of partially purified plasmid DNA in HEK293EBNA suspension cell culture to produce recombinant protein, using Green Fluorescent Protein (GFP) as a reporter.

Similar experiments to assess the transfectability of partially purified DNA preparations in HEK293 suspension

were carried out in an agitated 12 well plate system. The transfection itself was carried out as described in example 3. Cells were cultivated in Pro293Scom media (Biowhitaker, Walkersville, USA) in spinner cultures to densities of around 1 - 1.5 x 10^6 cells/ml (cells were counted both by trypan blue and packed cell volume methods). Cells were harvested by centrifugation and seeded in 12 well plates at a density of 500,000 cells/ml/well and incubated at 37°C, 5% CO_2 for 2 hours on an orbital shaker at 200rpm prior to transfection. Medium exchange was substituted by medium addition, 600 µl were added to the 1.1 ml volume in the 12 well plate in order to dissolve the precipitate.

Figure 5 shows a comparison of GFP expression 3 days post transfection after parallel transfections of partially purified and pure preparations of pEGFPN1. GFP signal is shown for intact cells and after cell lysis by the addition of 1ml 1% Triton X-100 in PBS. As with the adherent cell system there is no difference in expression between transfections with pure and crude DNA preparations.

Example 5. Transfection of partially purified plasmid DNA preparation on a reactor scale to produce recombinant protein.

Reactor transfections were carried out in order to demonstrate the application of partially purified plasmid DNA preparations in a large-scale transfection system. Transfections were carried out in a controlled 2l stirred tank vessel fitted with a single lightnin A315 impellor (Applikon). Oxygen and pH probes were calibrated, after

which the reactor was sterilized by autoclaving the whole reactor containing 1l of 0.1x PBS. Cells were cultivated in spinner cultures as described in example 4. After counting and viability estimation the appropriate volume of cells was centrifuged (1,000rpm, 5 minutes). Cells were resuspended in fresh medium, feme + 2% fcs, to give a concentration of 1 x 10^6 cells/ml. 300ml of this suspension were added to 300ml of pre-warmed medium (feme + 2% fcs) to give a concentration of 5 x 10^5 cells/ml in the vessel. Parameters were stirrer speed 200 rpm, temperature 37°C, pO2 35% (relative to air), pH 7.1. Cells were incubated in the reactor for 2 hours prior to transfection, 30 minutes before transfection the pH setpoint was shifted to the optimum for transfection, 7.4. Precipitate was formed by the addition of 15ml 2x Hepes Phosphate buffer (example 3) to each of two volumes of 15ml 250mM $CaCl_2$ containing 750µg plasmid DNA and mixing by pipetting up and down several times. The precipitate was taken up in a 50ml syringe after a 3 minute incubation time and injected over a septum fitted in the head plate of the reactor. At this point the pH control was switched off. 4 hours post transfection, 400ml of medium was added to the reactor to dissolve the precipitate, the pH control was then set to 7.1. Samples were taken at this point (blank) and subsequently every 24 hours after transfection. GFP signal was measured in 12 well plates as described for examples 3 and 4. Figure 6 shows a comparison between two reactors, transfected with (i) a pure preparation of pEGFPN1 and (ii) crude prep 1 pEGFPN1. In this experiment GFP expression was higher after transfection with the crude preparation than the pure preparation. Expression of GFP is presented in fig. 6 as in situ fluorescence. Similar results (not

shown) have been obtained by measuring GFP released into the medium after cell lysis with the addition of Triton X - 100. Expression of GFP in the reactor transfected with crude DNA again results in good transient expression, comparative to a reactor transfected with pure DNA.

Example 6. Demonstration of the tolerance limits of RNA in the transfection of DNA preparations by the calcium phosphate co-precipitation method. Demonstration of improvement/restoration of transfectability of partially purified DNA preparations by treatment with RNase A directly before transfection.

By adding back crude RNA extracted by ammonium acetate precipitation to pure DNA it was possible to work out when in the adherent and suspension systems a limitation in transfectability of the HEKEBNA cell line is reached. Bacterial RNA was extracted from the ammonium acetate precipitate, obtained during a DNA extraction of pEAKeGFP (similar to that described in examples 1 and 2) by:

(a) resuspending the RNA pellet from the ammonium acetate precipitation in TE;
(b) precipitating the RNA from (a). with isopropanol (0.6 volumes);
(c) centrifuging this at 4,000g, discarding the supernatant; and
(d) washing the RNA pellet in 70% ethanol, briefly drying the RNA pellet and resuspending in TE, pH 8.0.

RNA was analysed on a 1% Agarose gel and quantified by the ribogreen assay (Molecular Probes, Leiden, Netherlands). DNA was transfected at 2.5 µg per well in

adherent and suspension cell systems, control transfections were compared with transfections in which RNA was added in increasing amounts. In experiments using the construct pEAKeGFP, 11.7kb (pEGFPN1 (Clontech, Palo Alto, CA) inserted into the PEAK8 vector (Edge Biosystems, Gaithersburg, MD)), the addition of RNA in the transfection of HEKEBNA cells in suspension had no deleterious effect on GFP expression up 20 µg RNA (Figure 7a). A clear drop in expression was observed upon the addition of 40 µg RNA to the transfection cocktail. Therefore the limitation occurs (under the conditions of transfection, 2.5 µg DNA) between an 8 and 16 fold excess of crude RNA over DNA for suspension cells. In the adherent system there is no limitation at 5.0 µg (a two-fold excess of RNA to DNA) in the transfection. However a limitation is present at 10 µg RNA (a four-fold excess of RNA to DNA) (Figure 7b).

The RNA extract was added back to a further series of co - transfections of two pure plasmids preparations which encode anti Rhesus D IgG (a secreted protein). LH1 (light chain anti RhD IgG construct inserted into PEAK8 vector, 10.251kb) and LH2 (heavy chain anti RhD IgG construct inserted into PEAK8 vector, 11.439kb), were transfected in a previously determined optimum ratio of 30:70 (LH1: LH2) at 2.5 µg DNA per well. Transfection in the absence of RNA was compared to transfections with increasing amounts of added RNA. Expression of IgG in the supernatant (measured by ELISA, 5 days post transfection) was used as a measure of transfectability. In the suspension system (described in example 4), no negative effect on transfection was observed by the addition of RNA over the range tested, up to 10 µg added (Figure 8a).

In the adherent system a significant decrease in expression is observed between 5 and 10 µg RNA per well (as for the transfection of pEAKeGFP), figure 8b.

Additionally, crude preparations (prepared as described in examples 1 & 2) of the light and heavy chain plasmids were co - transfected in comparison and in combination with "pure" DNA preparations. The LH1 preparation contained an RNA concentration of 1.2 µg RNA per µg DNA (typical preparation). The preparation of LH2 (heavy chain plasmid) used had a particularly high RNA content, (RNA 3.52 µg/µg DNA). Figure 9 shows IgG expression measured in the supernatant 5 days post transfection. In the adherent system, the co-transfection of "pure" preparations of both LH1 and LH2, resulted in higher expression than that achieved by co-transfection of the two crude preparations (total RNA amount 7 µg/well, DNA amount 2.5 µg/well). Transfection with a combination of pure light chain plasmid prep, LH1, with the crude heavy chain prep, LH2, (RNA amount 6.16 µg/well, DNA 2.5 µg/well) resulted in expression slightly higher than that achieved with two pure preparations. This result confirms what is observed in experiments where RNA was added back to pure DNA preparations, a limitation occurs in the adherent system between 5 and 10 µg RNA/well. In addition both crude light (LH1) and heavy chain (LH2) preparations were treated with RNase A (Sigma, St. Louis, USA) before transfection. After a preliminary small scale digest to evaluate the minimum quantity of RNase A needed for RNA removal, digests of crude LH1 and LH2 were performed. This was done by the addition of 4 µl of RNase A at a concentration of 500 µg/ml to 1ml of the DNA preparation, to reach a concentration in the digest of 2 µg/ml RNase

A, (this corresponds to 0.08 U/ml). Transfection of the RNase digested preparation yielded transient IgG expression, higher than that obtained after transfection of pure DNA.

Figure 10 shows a comparative experiment in small-scale suspension culture (transfection conditions as example 4). An increase in the crude DNA fraction (pure LH1: crude LH2) enhanced the expression of IgG, the best expression was observed in the co-transfection of the two crude DNA preparations. Interestingly treatment of these preparations with RNase A had a positive effect on transfection when pure and crude preparations were mixed, the same expression was observed for crude and crude RNase treated DNA preparations.

Figure 11 shows the effect of RNase treatment on a poorly transfectable crude DNA preparation of pEAKeGFP, after transfection in HEK293EBNA adherent cells. Despite the negative effect on expression (at 2.5 µg/ml), after the transfection of this particular crude preparation, treatment with Rnase, (before transfection), restored GFP transient expression to a level comparable to that observed after transfection with a pure preparation of pEAKeGFP. Crude pEAKeGFP was extracted from material equivalent to 3l fermentation broth harvested at an OD600 of 4.0. Fermentation was carried out in LB medium, essentially as in examples 1 and 2, with the addition of a second ammonium acetate precipitation.

Comment on Example 6

The majority of preparations that have been produced by the extraction method described have been highly transfectable with no further treatment, with the exception of the named preparations of LH2 and pEAKeGFP given in example 6. Moreover transient expression can be fully restored in otherwise poorly transfectable crude preparations (e.g. which transfect poorly either as a result of a particular RNA contamination (figure 11), or as a result of an excessive concentration of RNA in the preparation for a particular application, i.e. in the transfection of adherent cells (figure 9)) by treatment with RNase.

Surprisingly the presence of RNase has no observable negative effect on transfection, which is fortuitous as RNase is heat stable and very difficult to remove, so it is useful to be able to leave it in the DNA preparation for transfection.

Positive effects on transfection efficiency have been observed (e.g. by Tatsuka et al. and in the examples above) in the presence of RNA, or in the present work after the digestion of RNA-containing DNA preparations with RNase before transfection. If RNA, free nucleotides or digested RNA fragments have a positive effect on transfection, we have not been able to observe these effects by the addition of RNA back to pure DNA, or the addition of an RNase digest to pure DNA.

Example 7 - Transfection of Mammalian Cells

NIH-3T3 cells (ATCC) were cultivated in adherent mode in DMEM with Glutamax-I in the presence of 10 % FBS. After

trypsinisation, 4 X 10^6 cells were resuspended in 350µl of growth media in an electroporation cuvette, mixed together with 10µg plasmid DNA (pEGFPN1) and 50µg carrier DNA (salmon sperm). Cells were pulsed once at 960µF and 250V at room temperature with a Gene Pulser II (BioRad). Cells were left to recover at room temperature for 15 minutes before the addition of 5ml of growth media and plating in a 10cm culture dish. Transfection efficiency was determined by counting the number of positive cells in a defined area of the dish and correlating this with the number of total cells in this area using a fluorescence microscope. The number of cells counted per estimation lied between 100 and 300 and at least 5 counts were performed for each condition. Data in table 1 show a comparison between the transfection of purified DNA (see previous sections), crude DNA and crude DNA digested with RNase A. The transfection efficiency was between 5.36 and 6.01 % for each of the 4 preparations transfected.

DNA Preparation	Mean & cells transfected
Pure 1.	5.46 +/ -1.99
Crude 1.	6.02 +/ -2.11
Crude 2.	5.43 +/ -1.17
Crude 2 + RNase	5.36 +/ -2.77

Table 1. Comparison of transfectability of NIH 3T3 cells by electroporation of pure, crude or curde treated plasmid (pEGFPN1) preparations. Transfectability measured as percentage of cells transfected, based on visual analysis.

Example 8 - Characterization of partially purified plasmid DNA

Alkaline lysis is followed by three successive precipitations, two isopropanol precipitations, interspersed with an RNA selective precipitation, employing ammonium acetate and a final wash in 70 % ethanol, to yield partially purified plasmid DNA. Figure 12 shows the appearance of three pEGFPN1 preparations on a 1 % agarose gel by comparison to a pure DNA preparation. Crude DNA preparations have a high degree of supercoiled structure; treatment with RNase A removes the dominant RNA band in the lower part of the gel, yielding a DNA quality which corresponds closely to pure DNA (not shown). The concentrations of the principal contaminants (LPS, protein, RNA) are summarised in Table 2, for the three preparations at a plasmid DNA concentration of 1 mg/ml. Table 2 shows that the three batches contained similar amounts of RNA (respectively 1.15, 1.19 and 1.63 mg/ml). LPS concentrations showed more preparation to preparation variability, ranging from 3×10^3 (prep 3) to circa 5×10^5 EU/ml (prep 2). SDS PAGE gel electrophoresis yields no detectable protein bands in either the pure of crude DNA extracts by coomasie or silver staining (RNase and DNase treatments were used before silver staining in order to remove non specific background from nucleic acids). Likewise upon nucleic acid digestion, protein was undetectable by a standard Biorad assay (<50 µg/ml).

Example 9 - Kinetics of DNA Calcium Phosphate precipitate formation

The first step towards effective transfection by the calcium phosphate method is the formation of an adequate DNA calcium phosphate precipitate complex. We used a turbidimetric assay, described by Jordan et al. Nucleic Acid Research 24, 596-601 (1996) to assess calcium phosphate precipitate formation. T his assay gives some information about the character of the forming precipitate. Using this approach we compared differences between pure and crude preparations of DNA (pEGFPN1) on precipitate formation. Additionally, the contribution of RNA on precipitate formation was assessed in experiments using crude DNA extracts treated with RNase A. Finally, RNase digested preparations were dialysed 2x against TE (8,000 NMWCO membrane) removing RNase digestion products.

At a plasmid DNA concentration in the precipitate cocktail of 25 µg/ml (corresponding to 2.5 µg/ml culture volume), OD readings, measured after 1 minute, were slightly higher in precipitates formed with crude extracts than those formed using column purified DNA's (Fig 13a). RNase treatment of crude extracts 1 and 2, as well as RNase followed by dialysis had little effect on precipitate formation in precipitates formed with extracts 1 and 2, which were similar to pure DNA. Digestion of extract 3 with RNase lead to the formation of a more abundant precipitate, this increase in precipitate formation was significantly reduced after dialysis. This initial increase in the RNase digested extract may result from the presence of a greater concentration of RNA digestion products in extract 3 (see Table 2), which may enhance precipitate formation. Precipitate formation may also be additionally affected

by the presence of other molecules even at low concentrations, therefore the influence of such unknown factors cannot be ruled out.

	RNA (mg/ml)	LPS (EU/ml)	Protein (mg/ml)
Extract 1.	1.15	194,200	<.05
Extract 2.	1.19	500,200	<.05
Extract 3.	1.63	3,000	<.05

Table 2. Principal contaminants of crude DNA extracts prepared by alkaline lysis and 3 step precipitation. All DNA preparations were diluted to 1mg/ml based on picogreen quantification.

Example 10 - Demonstration of the transfectability of crude plasmid DNA preparations in adherent and suspension adapted HEK293 EBNA cells.

In order to study if crude DNA extracts could be reproducibly transfected, the three batches of crude pEGFPN1 (shown in Fig. 12) were transfected in adherent cells in parallel. Transfections with pure DNA preparations served as a control. Adherent HEK293 cells were transfected at a previously determined optimal DNA concentration (based on experiments with pure DNA preparations) of 2.5 µg/ml culture volume. All transfections were performed in triplicate. GFP expression from the vector pEGFPN1 was measured as in-situ fluorescence, after 1, 2 and 3 days.

At 2.5 µg DNA/ml culture volume, transient GFP expression from crude DNA extracts was comparable to that obtained upon transfection of pure pEGFPN1 (Fig 14a). Similarly,

little difference was observed between crude extracts and pure preparations of pEGFPN1 at a sub optimal DNA concentration of 1 µg DNA/ml culture volume (data not shown). With crude DNA extracts we obtained recombinant protein synthesis similar to that seen in cells transfected with pure DNA.

To remove RNA, extracts were treated with RNase A directly before transfection, removal of RNA was checked by analysis on a 1 % agarose gel (not shown). The presence of the enzyme RNase in the cocktail did not affect transfection efficiency. This was verified by transfection of pure DNA, to which RNase had been added (Fig. 14a). In addition to RNase treatment we dialysed a fraction of the RNase treated extract two times against TE buffer using a membrane with a NMWCO of 8,000. Transfection of such preparations yielded similar expression levels to non-dialysed DNA extracts. Similar effects were seen again at sub-optimal DNA concentration for transfection, 1.0 µg/ml culture volume (data not shown). Transient GFP expression relative to expression from the reference transfection of pure DNA was similar over 3 days in most cases. Therefore, we can conclude that even in plasmid preparations in which the RNA concentration is of the order of 1-2 µg RNA/µg plasmid DNA, treatment with RNase A or RNase A followed by Dialysis has little overall effect on transfection.

Conclusions

The transfection of "crude" partially purified plasmid DNA preparations is possible and partially purified DNA

preparations are expected to be suitable also for other r-DNA applications.

RNA becomes a limiting factor only at high concentrations in transfection, (using the cell line tested and under the experimental conditions of transfection used). If RNA is limiting the negative effect can be overcome by the treatment of the preparation with RNase A.

A scalable method has been developed for DNA extraction to reproducibly yield a preparation suitable for transfection. This is achieved by alkaline lysis and a series of easily performed precipitations. Plasmid DNA stayed intact despite the relatively harsh mixing conditions applied in the stirred tank reactor during alkaline lysis, a process often described as delicate. The method offers significant benefits over other large - scale methods in that it requires little specialized equipment or reagents. In addition it is quick and relatively cost effective. It significantly benefits large-scale DNA delivery processes for the production of commercial products, such as scalable protein expression by transient transfection.

A beneficial effect may be seen in terms of protein expression upon transfection of crude DNA and upon crude DNA treated with RNase before transfection.

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Relevant patents

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Wurm F. M. and Bernard A. R. - Large - scale transient expression in mammalian cells for recombinant protein products. Current Opinion in Biotechnology. 10: 156 - 159. (1999).

On-line transfection guides

Promega - Transfection guide, www.promega.com.

Qiagen - The Art of Efficient Transfection, www.qiagen.com.

All references are incorporated herein by reference in their entirety and for all purposes.

CLAIMS:

1. A method of preparing from bacterial cells a plasmid preparation containing at least 10mg of DNA, the method comprising the steps of:

(a) lysing the bacterial cells containing a plasmid;

(b) precipitating bacterial cell debris;

(c) separating the cell lysate, containing plasmid DNA, from the precipitated cell debris; and

(d) precipitating the plasmid DNA from the cell lysate, separating the precipitated plasmid DNA from the cell lysate and resuspending the plasmid DNA.

2. A method according to claim 1 wherein step (a) is an alkaline lysis step.

3. A method according to claim 2 wherein the alkaline lysis step is performed at between pH 12 and pH 13.

4. A method according to any one of the preceding claims further comprising the step of

(e) selectively precipitating RNA and/or cell protein from the cell lysate and/or resuspended plasmid DNA and separating the supernatant, containing the plasmid DNA, from the precipitate.

5. A method according to claim 4 wherein step (d) is repeated after step (e).

6. A method according to any one of the preceding claims further comprising the steps of treating the plasmid preparation to further remove RNA.

7. A method according to claim 6 wherein the further RNA is removed using RNase.

8. A method according to claim 7 wherein the products resulting from the RNase treatment are removed by a dialysis step.

9. A method according to any one of the preceding claims wherein the plasmid preparation contains at least 5mg of plasmid DNA per litre of bacterial cell culture solution.

10. A method according to any one of the preceding claims further comprising the step of transfecting a eukaryotic cell with the plasmid preparation resulting from the previous method steps.

11. A method according to claim 10 further comprising the step of culturing the eukaryotic cell so as to express a protein encoded by the transfected plasmid.

12. A method according to claim 11 further comprising the step of isolating and/or purifying the expressed protein.

13. A plasmid preparation as prepared by a method according to any one of claims 1 to 9.

14. A eukaryotic cell transfected by a plasmid prepared by a method according to claim 10.

15. A method of transfecting a eukaryotic cell, having previously produced a plasmid preparation according to the method of claims 1 to 9, the method comprising the steps of transfecting the cell with said plasmid preparation wherein said cell is transfected with a plasmid contained in the plasmid preparation in the presence of contaminants contained within the plasmid preparation.

16. A method according to claim 15 wherein the contaminants include RNA.

17. A method according to claim 15 wherein the contaminants include an RNase.

18. A plasmid DNA preparation which is transfectable into eukaryotic cells, the plasmid preparation containing at least 10mg DNA, wherein the preparation is a crude preparation containing other cellular products.

19. A plasmid DNA preparation according to claim 18 wherein the cellular products include RNA.

20. A plasmid DNA preparation according to claim 19 wherein the RNA is present in a ratio to DNA of between 20:1 to 1:20.

21. A plasmid DNA preparation according to any one of the previous claims wherein the cellular products include protein and LPS.

22. A plasmid DNA preparation according to any one of claims 18 to 21 which further contains an RNase.

23. A method of transfecting a eukaryotic cell, said method comprising the steps of exposing the cell to a plasmid preparation according to any one of claims 18 to 22 under conditions suitable for transfection to occur.

Fig.1.

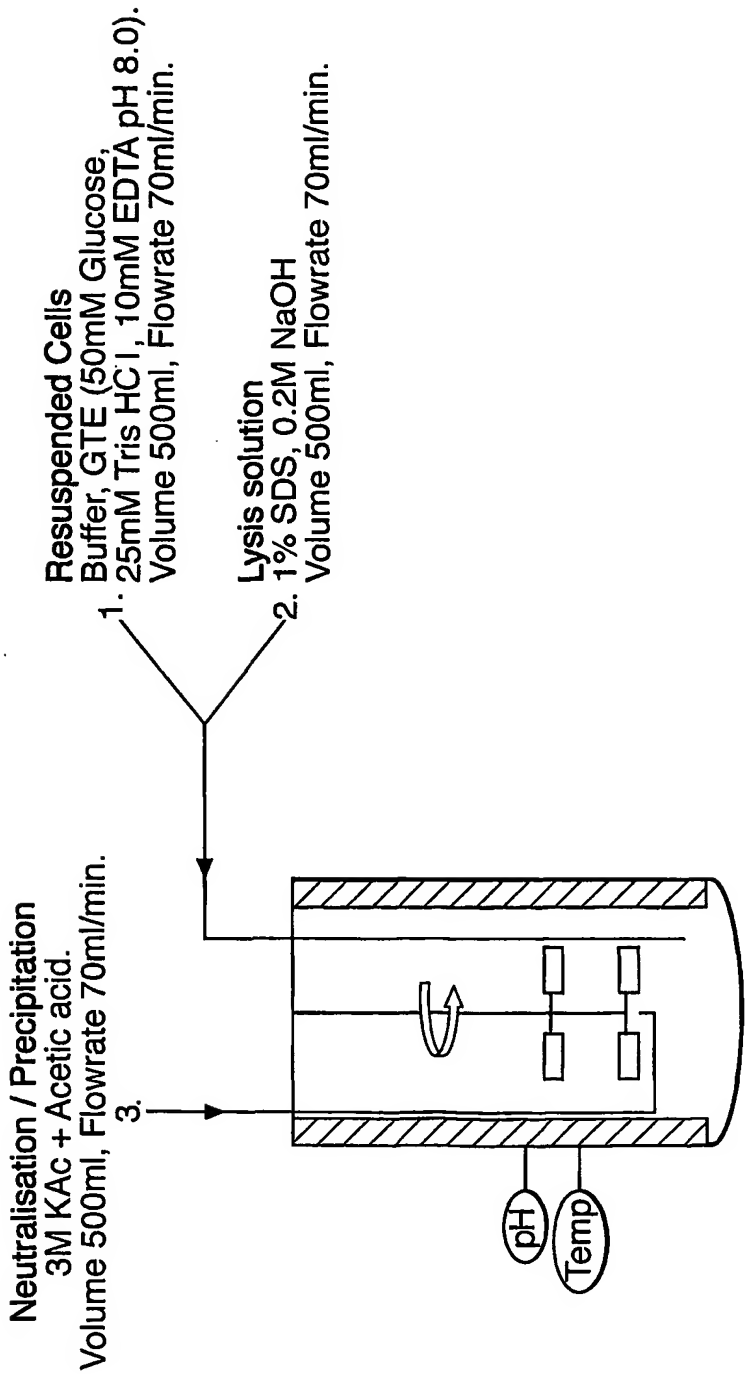
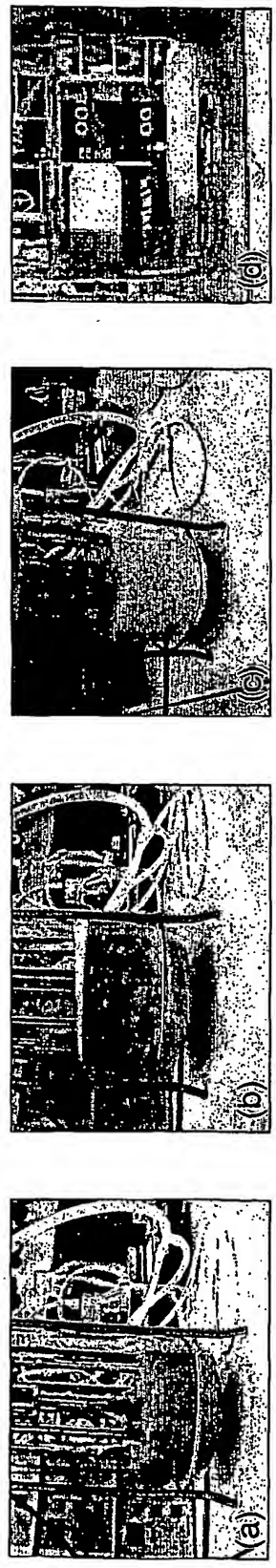

Resuspended Cells
Buffer, GTE (50mM Glucose,
1. 25mM Tris HCl, 10mM EDTA pH 8.0).
Volume 500ml, Flowrate 70ml/min.
Lysis solution
2. 1% SDS, 0.2M NaOH
Volume 500ml, Flowrate 70ml/min.
Neutralisation / Precipitation
3M KAc + Acetic acid.
Volume 500ml, Flowrate 70ml/min.
3.
pH
Temp

Fig.2.



M - 1Kb ladder.
1 - Pure DNA prep (M & N)
2 - Crude prep 1.
3 - Crude prep 2.
4 - Crude prep 1. + RNase

Figure 3

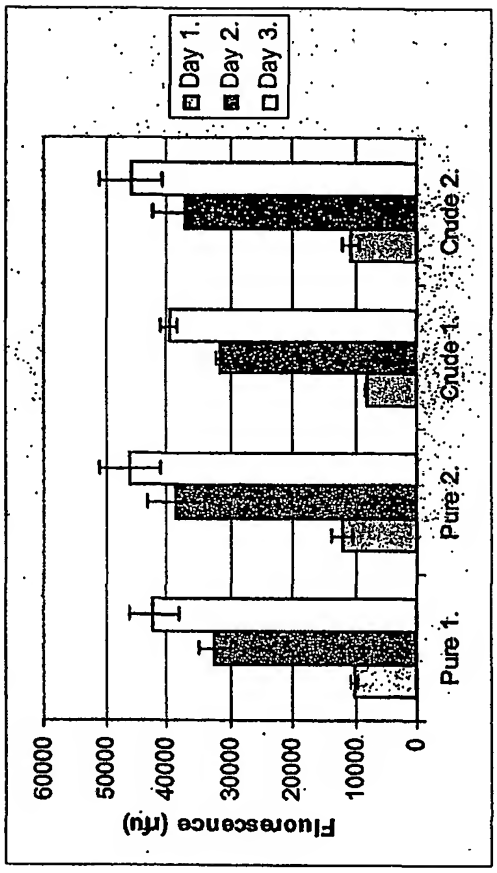

Day 1.
Day 2.
Day 3.
Crude 2.
Crude 1.
Pure 2.
Pure 1.
Fluorescence (rfu)
60000
50000
40000
30000
20000
10000
0

Figure 4

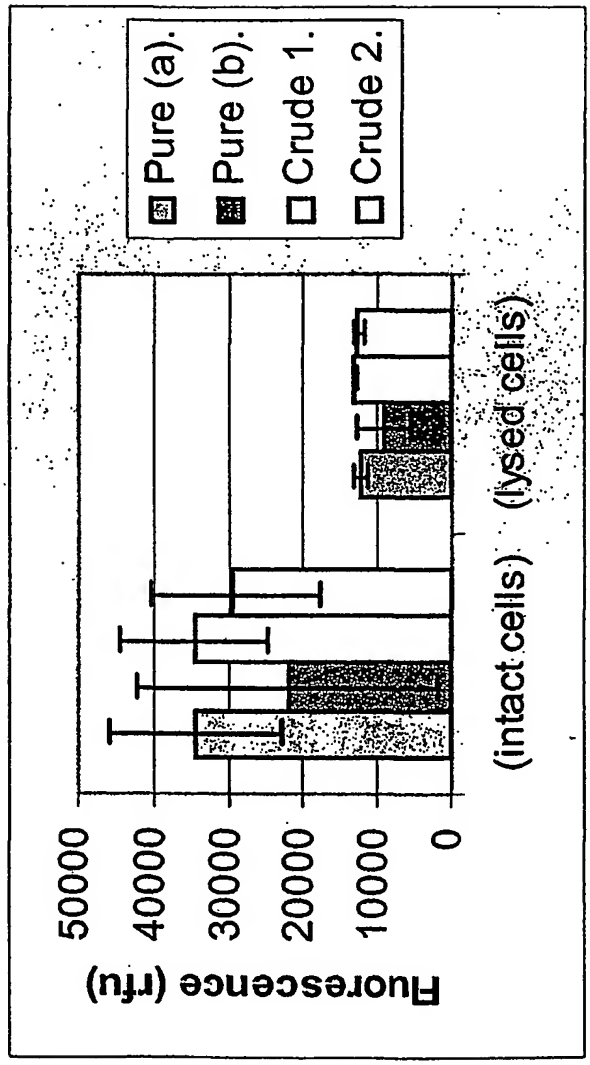

Pure (a).
Pure (b).
Crude 1.
Crude 2.
Fluorescence (rfu)
50000
40000
30000
20000
10000
0
(intact cells)
(lysed cells)

Figure 5

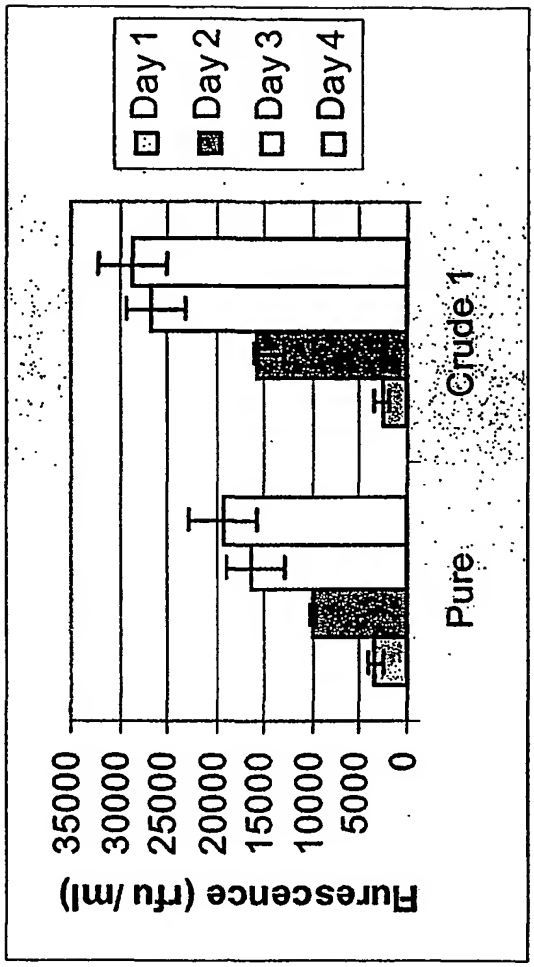

Day 1
Day 2
Day 3
Day 4
Crude 1
Pure
Fluorescence (rfu /ml)
35000
30000
25000
20000
15000
10000
5000
0

Figure 6

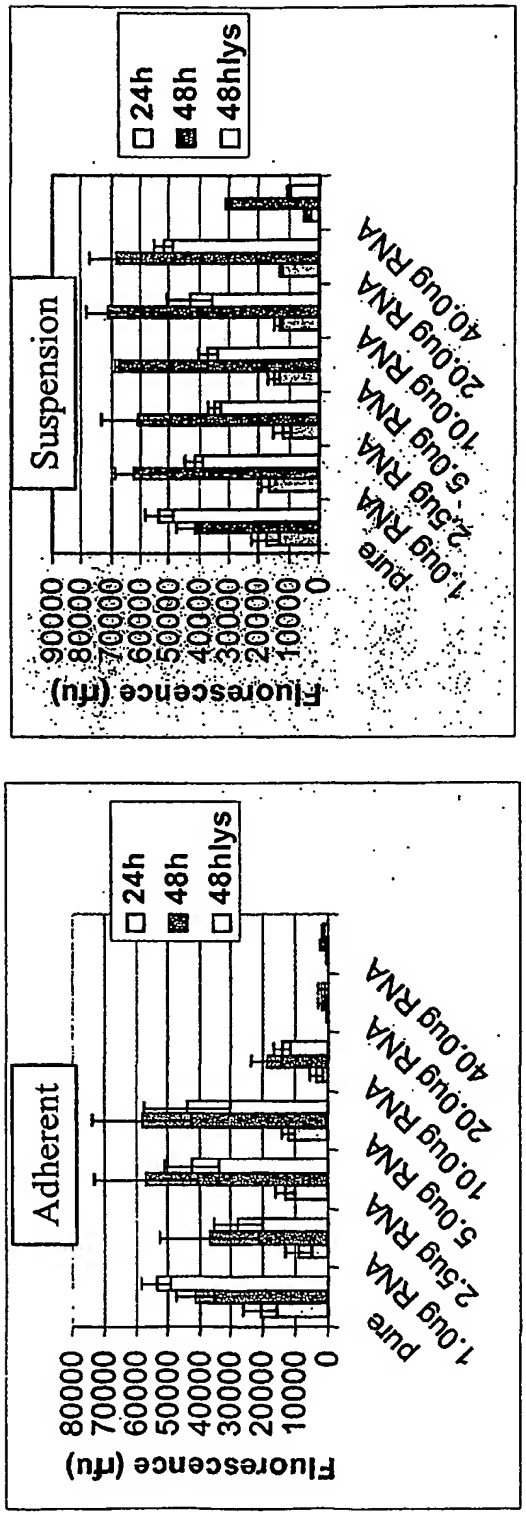

Suspension
24h
48h
48hlys
Fluorescence (rfu)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
pure
1.0ug RNA
2.5ug RNA
5.0ug RNA
10.0ug RNA
20.0ug RNA
40.0ug RNA
Adherent
24h
48h
48hlys
Fluorescence (rfu)
80000
70000
60000
50000
40000
30000
20000
10000
0
pure
1.0ug RNA
2.5ug RNA
5.0ug RNA
10.0ug RNA
20.0ug RNA
40.0ug RNA

Figure 7

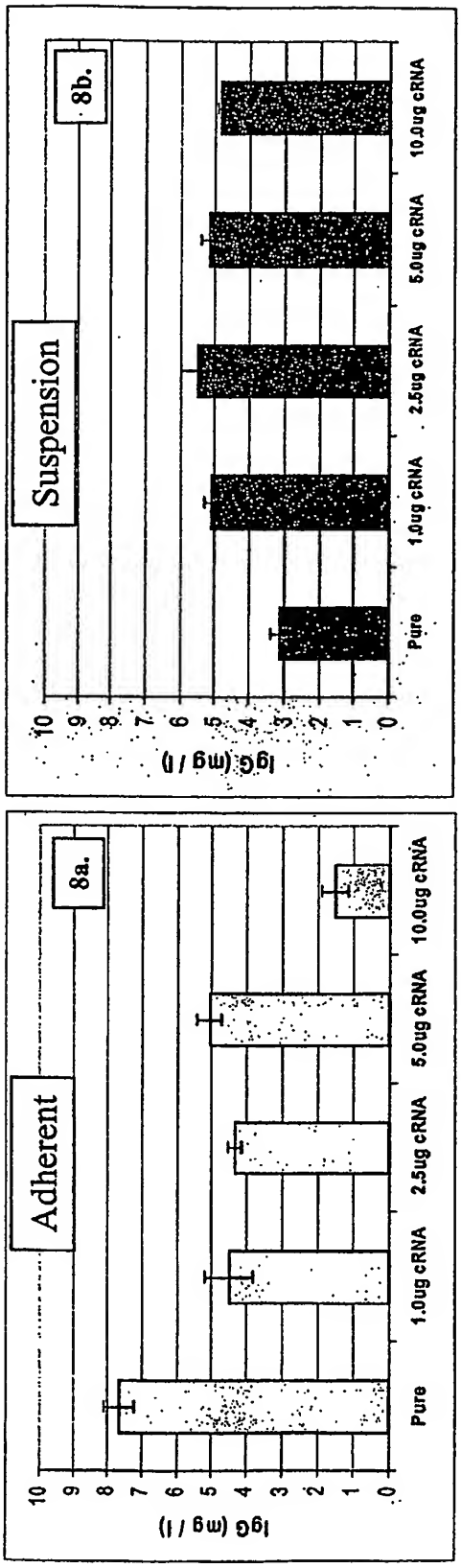

8a.
Adherent
IgG (mg / l)
0
1
2
3
4
5
6
7
8
9
10
Pure
1.0ug cRNA
2.5ug cRNA
5.0ug cRNA
10.0ug cRNA
8b.
Suspension
IgG (mg / l)
0
1
2
3
4
5
6
7
8
9
10
Pure
1.0ug cRNA
2.5ug cRNA
5.0ug cRNA
10.0ug cRNA

Figure 8

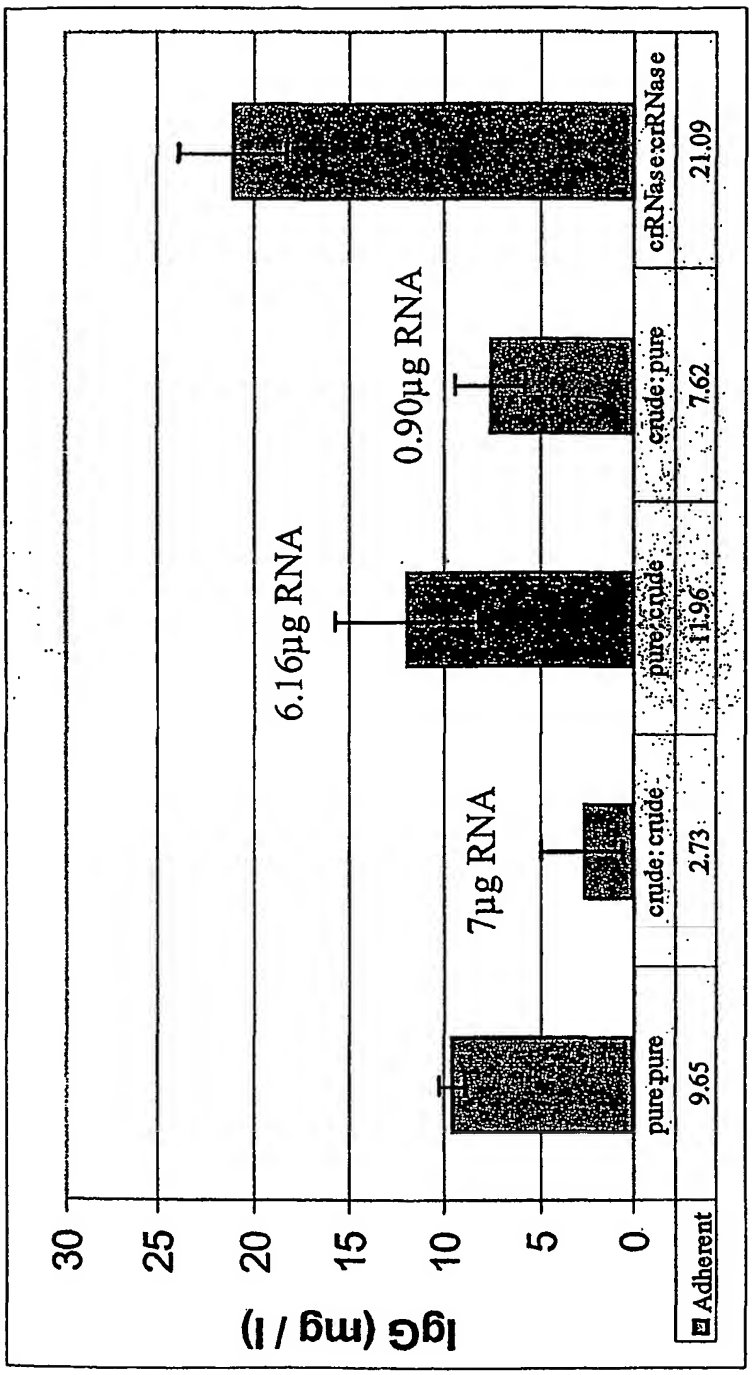

IgG (mg / l)
30
25
20
15
10
5
0
6.16µg RNA
0.90µg RNA
7µg RNA
pure:pure
crude:crude
pure:crude
crude:pure
crRNase:crRNase
Adherent
9.65
2.73
11.96
7.62
21.09

Figure 9

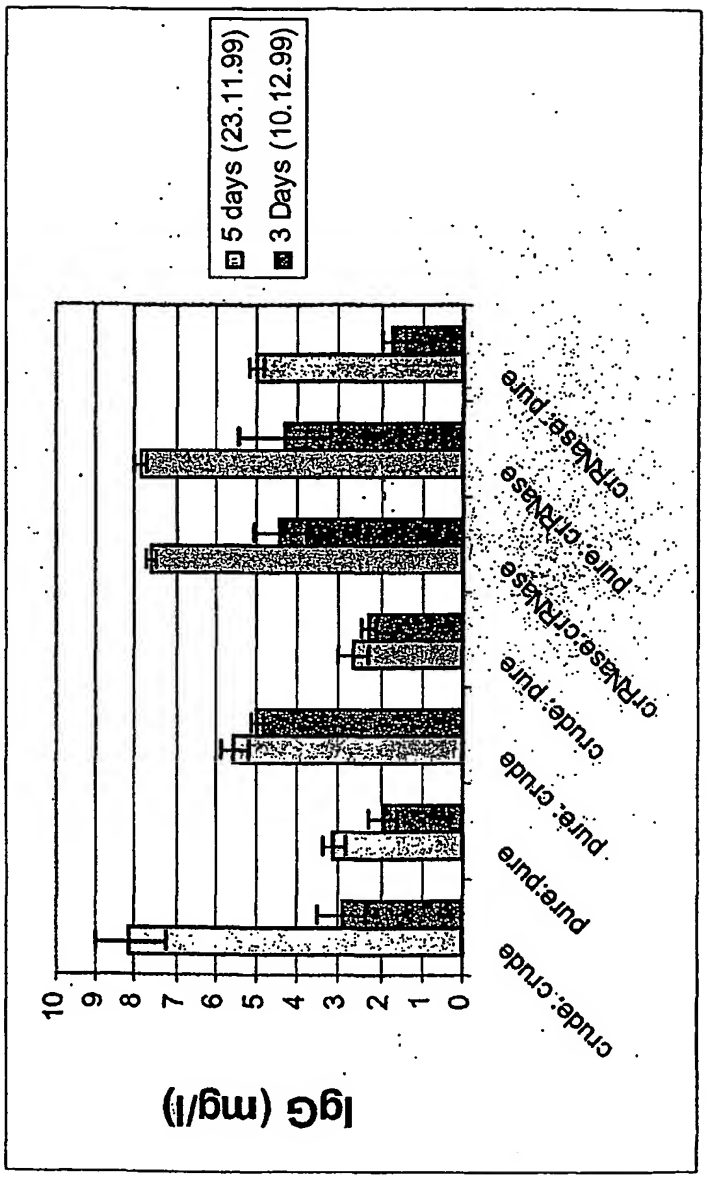
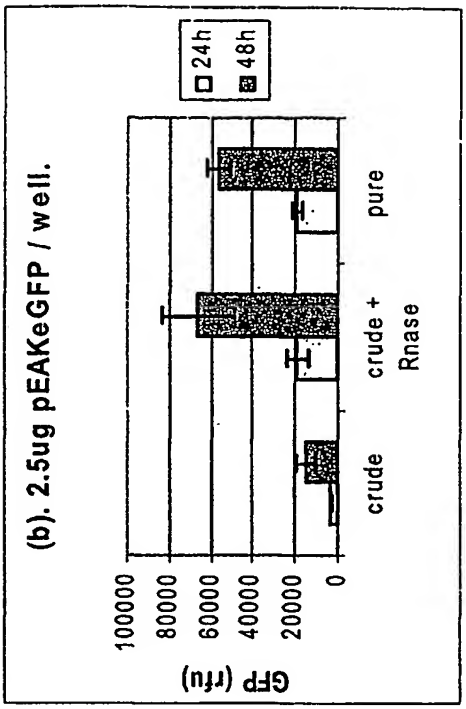

5 days (23.11.99)
3 Days (10.12.99)
IgG (mg/l)
0
1
2
3
4
5
6
7
8
9
10
crude:crude
pure:pure
pure: crude
crude: pure
crRNase:crRNase
pure: crRNase
crRNase: pure

Figure 10


(b). 2.5ug pEAKeGFP / well.
24h
48h
GFP (rfu)
100000
80000
60000
40000
20000
0
crude
crude + Rnase
pure

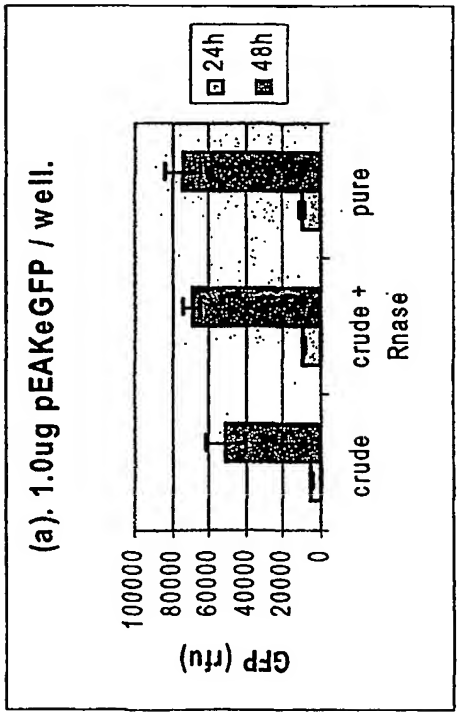

(a). 1.0ug pEAKeGFP / well.
24h
48h
GFP (rfu)
100000
80000
60000
40000
20000
0
crude
crude + Rnase
pure

Figure 11

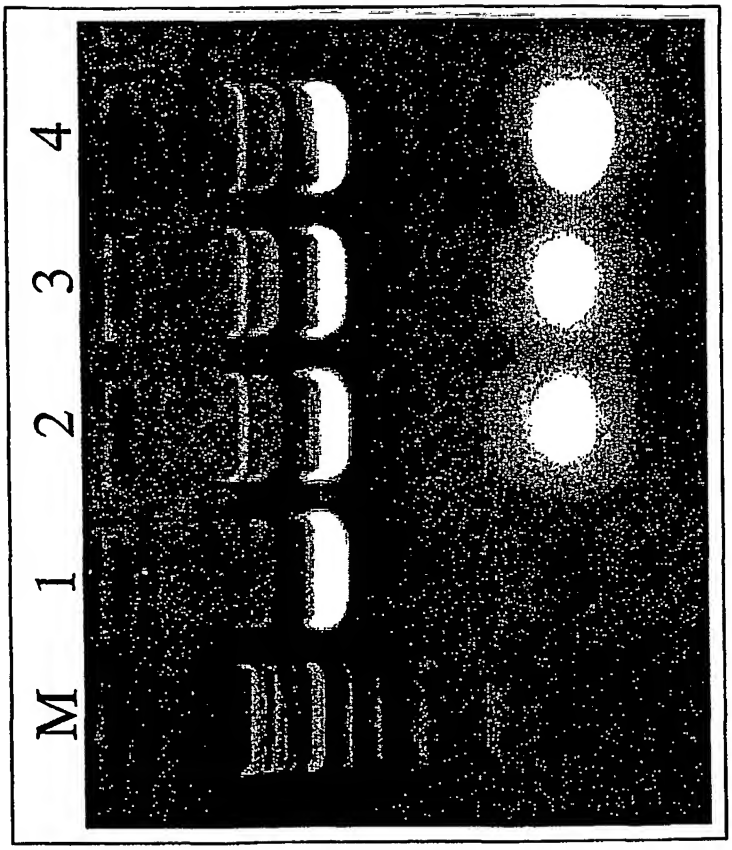


Figure 12

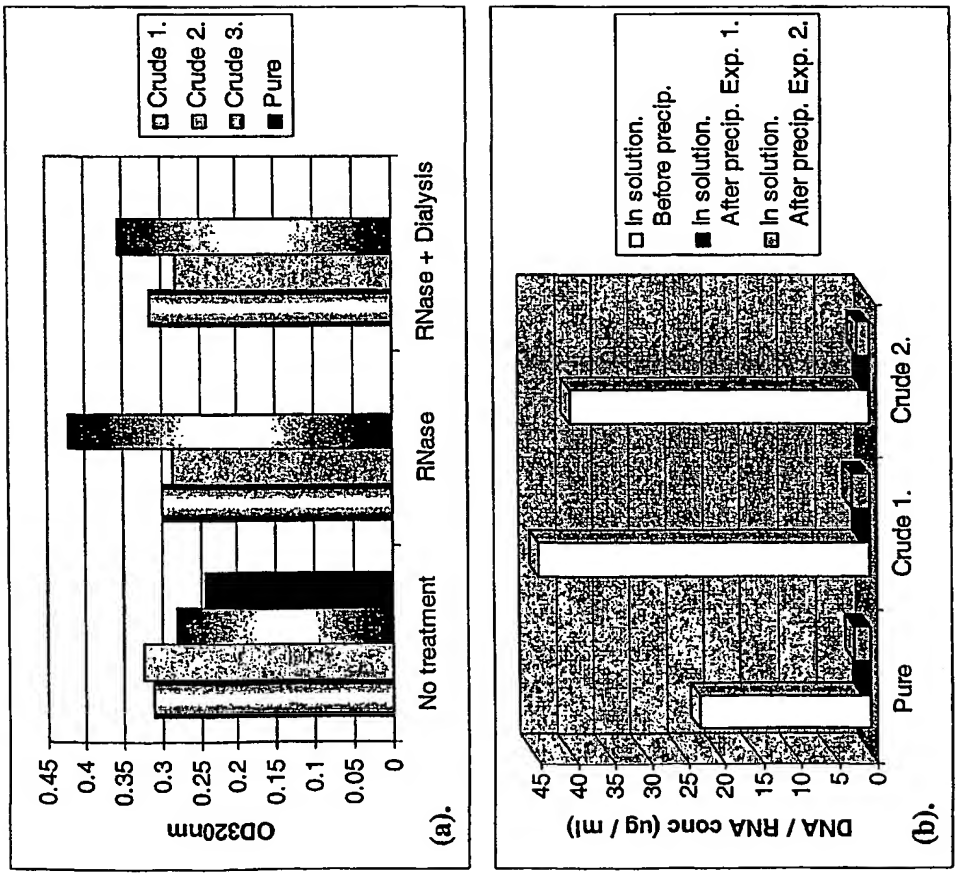

Crude 1.
Crude 2.
Crude 3.
Pure
OD320nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
No treatment
RNase
RNase + Dialysis
(a).
In solution. Before precip.
In solution. After precip. Exp. 1.
In solution. After precip. Exp. 2.
DNA / RNA conc (ug / ml)
45
40
35
30
25
20
15
10
5
0
Pure
Crude 1.
Crude 2.
(b).

Figure 13

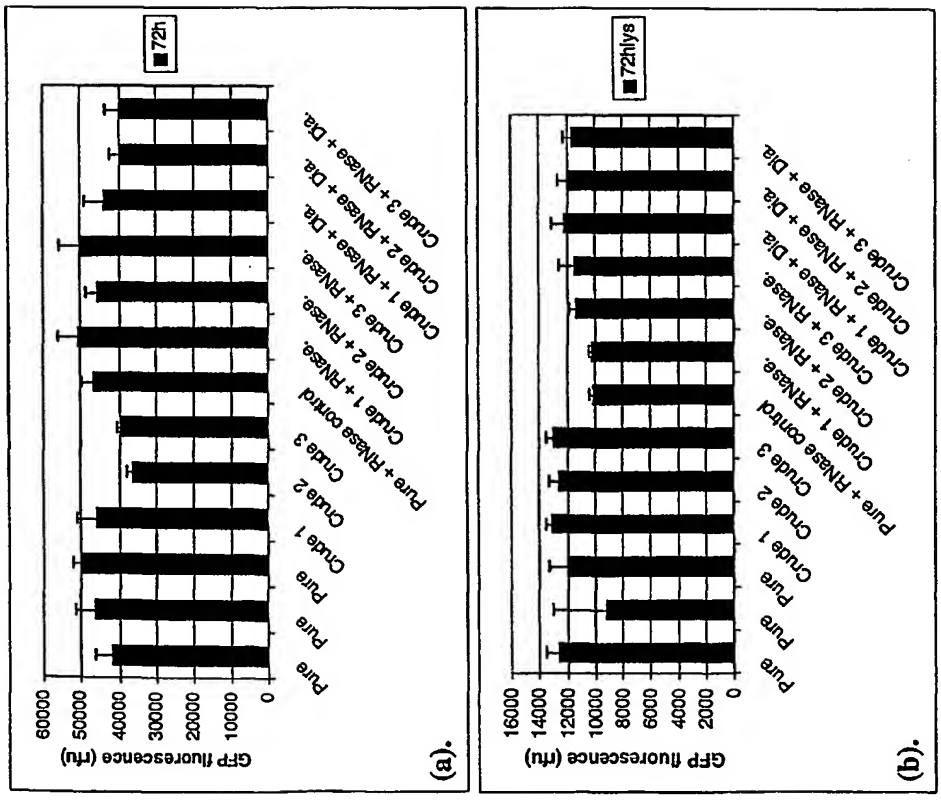

(a).
72h
GFP fluorescence (rfu)
60000
50000
40000
30000
20000
10000
0
Pure
Pure
Pure
Pure
Crude 1
Crude 2
Crude 3
Pure + RNase contrd
Crude 1 + RNase.
Crude 2 + RNase.
Crude 3 + RNase.
Crude 1 + RNase + Dia.
Crude 2 + RNase + Dia.
Crude 3 + RNase + Dia.
(b).
72hlys
GFP fluorescence (rfu)
16000
14000
12000
10000
8000
6000
4000
2000
0
Pure
Pure
Pure
Crude 1
Crude 2
Crude 3
Pure + RNase contcr
Crude 1 + RNase.
Crude 2 + RNase.
Crude 3 + RNase.
Crude 1 + RNase + Dia.
Crude 2 + RNase + Dia.
Crude 3 + RNase + Dia.

Figure 14